

DERIVATISED ANTIBODIES WITH EXPOSED CARBOHYDRATE CHAINS CAPABLE OF BINDING TO
IMMOBILISED IGG

Field of the Invention

The present invention relates to derivatised antibodies and their use in the diagnosis and treatment of conditions associated with immobilised IgG, specifically autoantibodies, and in particular to conditions associated with elevated levels of immobilised IgG, such as rheumatoid arthritis (RA). The present invention also relates to a method of producing the derivatised antibodies.

Background of the Invention

A central problem in clinical rheumatology is the need to define at presentation using non invasive techniques those patients who will go on to develop erosive joint disease. If such patients can be identified, then aggressive therapy can be targeted to them and more conservative therapy can be reserved for the remaining patients with RA who do not go on to erosive joint disease. This would improve the treatment of both groups, since early treatment in the erosive group can prevent the progression of disease and in the benign group reduce morbidity due to less frequent and severe drug reactions and toxicity.

A number of clinical parameters have been assessed for their ability to predict outcome of erosive joint disease. These include age, sex, RF status, genetic background (HLA) and serum agalactosyl IgG levels (see for example JP-A-5087814). However, prior studies have found that no single parameter is useful as a predictor. However, a combination of agalactosyl IgG levels, age of

onset, gender, functional assessments and RF titre predicts the course of RA correctly in 94% of patients.

Agalactosyl IgG is an IgG glycoform which is increased in patients with rheumatoid arthritis, tuberculosis, Crohn's disease, juvenile onset arthritis, sarcoidosis, type I insulin dependent diabetes, type II diabetes and a form of leprosy called erythema nodosum leprosum. Agalactosyl IgG is thought to cause pathology in the respective target tissues by a variety of mechanisms including:

(a) Tissue damage via the interaction of *in situ* immobilised agalactosyl IgG and the lectins mannose binding protein (MBP), tumour necrosis factor (TNF α) and transforming growth factor (TGF β). The tissue immobilised autoantibodies can bind these inflammatory mediators and trap them in the tissue, leading to tissue damage.

(b) Agalactosyl IgG is thought to cause systemic pathology by forming self-associating immune complexes.

The target organ or tissue for agalactosyl IgG action depends on the specificity of the autoantibody. In the case of rheumatoid arthritis, it is thought that autoantibodies against type II collagen are targeted to the joint. Cross-sectional studies of rheumatoid arthritis patients have demonstrated a frequency of only 30% for patients with detectable antibodies to type II collagen late in disease. In early RA the frequency may reach 70%. The problem of identifying patients likely to

develop erosive joint disease is complicated as three groups of rheumatoid arthritis patients can be classified by their anti-collagen autoantibody serum titres.

5 Group 1: Normal titre of anti-type II collagen antibodies.

Group 2: Elevated titre of anti-type II collagen antibodies which remain constant.

10 Group 3: Elevated titre of anti-type II collagen antibodies early in disease with a decrease to normal level later in disease. In this case, the decrease in the level of serum antibodies to collagen may include:

15 (a) Intrasynovial synthesis of antibodies to collagen (in contrast to systemic production);

20 (b) Removal of antibodies to collagen secondary to immune-complex formation; and/or,

(c) Serum skimming by immobilisation of antibodies to damaged cartilage surfaces.

25 Of the three groups described above, Group 3 will go on to get severe erosive disease and Group 1 mild disease. Therefore, serum antibody levels cannot discriminate between Group 1 and 3 once the levels of antibody in Group 3 has returned to normal.

30 While, the affected synovial joints of Group 3 patients

(in contrast to Group 1) are coated with anti-type II collagen antibodies of the agalactosyl IgG glycoform, joint biopsy is not a routine nor desirable procedure to detect these antibodies.

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Summary of the Invention

Broadly, the present invention provides derivatised antibodies, the antibodies being derivatised to expose carbohydrate chains so that the antibodies are capable of binding to immobilised IgG, such as agalactosyl IgG.

Thus, the derivatised antibodies can be employed in the diagnosis and treatment of conditions associated with immobilised IgG, such as rheumatoid arthritis (RA) (Group 3 versus Group 1 above). In particular, the present invention provides a non-invasive technique which can detect the immobilised IgG *in situ*, thereby assisting in the diagnosis or treatment of conditions associated with it. The present invention can help to avoid measuring the range of clinical parameters currently used to determine the likely course of RA.

Accordingly, in a first aspect, the present invention provides a derivatised antibody, the antibody being derivatised to expose carbohydrate chains so that the antibody is capable of binding to immobilised IgG, e.g. agalactosyl IgG. Preferably, the derivatised antibodies are also capable of self associating or "stacking" at the site of the immobilised IgG to assist in diagnosis or therapy of the patient by amplifying the concentration of the derivatised antibody that can be delivered to the site of the immobilised IgG. The present invention

further provides the above antibodies for use in a method of medical treatment.

5 The present invention is applicable to the diagnosis or treatment of conditions associated with immobilised IgG, and in particular agalactosyl IgG, such as autoimmune disorders including rheumatoid arthritis, juvenile arthritis, Crohn's disease, type I insulin dependent diabetes, type II diabetes, sarcoidosis, erythema nodosum
10 leprosum and tuberculosis. In a preferred embodiment, the present invention can be employed in the diagnosis of erosive joint disease by detecting the presence of tissue immobilised anti-type II collagen antibodies produced in rheumatoid arthritis patients susceptible to erosive
15 joint disease (Group 3).

In a further aspect, the present invention provides a method of producing a derivatised antibody, the method comprising treating a precursor antibody to expose
20 carbohydrate chains so that the antibody is capable of binding to immobilised IgG. Preferably, the method involves thiolating the antibody in the presence of carbonate, such as ammonium carbonate or sodium carbonate. In some embodiments of the invention, only a
25 fraction of the total derivatised antibodies can self associate or stack, referred to as the "imaging fraction" below. In this case, preferably the method additionally involves the step of separating this fraction of the derivatised antibodies, e.g. using Con A chromatography
30 or by pH elution of the derivatised antibody from a Protein A affinity column. In a preferred embodiment,

the precursor antibody has Fc carbohydrate chains which terminate with N-acetylglucosamine (GlcNAc) residues.

The proportion of such chains on the precursor antibody can be increased from naturally occurring levels by the use of β -galactosidase to remove terminal galactose residues. The imaging fraction of the derivatised antibodies should also contain Fab associated carbohydrate chains.

The method flips the internal carbohydrate chains, e.g. Fc carbohydrate chains, from their binding sites on the interstitial protein surface. The IgG is then chemically derivatised in a manner which prevents the carbohydrates from returning to their interstitial positions.

The binding between the immobilised IgG and the derivatised antibodies can be via the Fab carbohydrate chains of the derivatised antibodies binding to Fc binding sites or surfaces of the immobilised IgG and/or exposed Fab carbohydrate chains of the immobilised antibodies binding to Fc binding sites or surfaces exposed on the derivatised antibodies by the derivatisation reaction. Thus, in either case, the binding reaction is between a carbohydrate chain and a carbohydrate binding site on the antibody and is not linked to the immunological binding domain of the antibodies. In the case of agalactosyl IgG, as these antibodies lack galactosyl and sialic acid residues, this provides a binding site that can receive the available Fab carbohydrate chains of the derivatised antibodies. In preferred embodiment, the derivatised antibodies can

bind to themselves as well as binding with the immobilised IgG. This can happen if the flipping out of the carbohydrate chains opens up a binding site that is capable of binding carbohydrate chains on other antibodies (Fab, Fc etc). This stacking of the antibodies can provide a method of delivering the functional moiety to the locations in a patient where immobilised IgG concentrates, and also a method of concentrating the derivatised antibodies at these locations.

The precursor antibody can be polyclonal or monoclonal, and can be IgE, IgA, IgM (monomer), IgD or IgG. The production and purification of antibodies is described in detail below. In the examples below, the precursor antibody used is Sandoglobulin or CLB, polyclonal IgG mixtures.

In a further aspect, the present invention provides the use of a derivatised antibody for the preparation of a medicament for the treatment or diagnosis of a condition associated with immobilised IgG, the antibody being derivatised to expose carbohydrate chains so that the antibody is capable of binding to immobilised IgG.

In some embodiments, the derivatised antibody can be conjugated to a functional moiety, such as a label, toxin, drug, prodrug, effector or other moiety. Labels allow the antibody to be visualised *in vivo* or *in vitro*, indicating the presence and/or location of agalactosyl IgG. In a preferred embodiment, the label is a

radioactive label such as ^{99m}Tc . Where the functional moiety is a drug, prodrug or effector, the derivatised antibodies can deliver the functional moiety to locations in a patient's body where immobilised IgG is found and where the action of the drug, prodrug or effector is required.

Alternatively or additionally, the precursor antibody can itself have a therapeutic effect when delivered to locations in which immobilised IgG accumulates. By way of example, a derivatised antibody with specificity against an inflammatory mediator such as $\text{TNF-}\alpha$ could be used to ameliorate the effects of these substances at locations in a patient having elevated levels of immobilised IgG. In a further aspect, the present invention provides a method of diagnosing *in vivo* a condition associated with immobilised IgG, the method comprising (a) exposing a patient to derivatised antibodies, the antibodies being derivatised to expose carbohydrate chains so that the antibodies can bind to immobilised IgG and being labelled, and (b) using the label to detect the presence of the immobilised IgG. Conveniently, the derivatised antibodies can be administered to the patient by injection.

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more of the above mentioned antibodies in combination with a pharmaceutically acceptable carrier.

The present invention will now be described by way of

example and not limitation with referenc to the accompanying figures.

Brief Description of the Drawings

- 5 Figure 1 shows the anti-GlcNAc reactivity of immobilised Sandoglobulin or derivatised Sandoglobulin (HIG). Solutions containing various concentration of Sandoglobulin or HIG were added to microtitre wells coated with protein A. The top figure shows the anti-GlcNAc reactivity of the immobilised IgG or HIG after heating the plate 85°C for 10 minutes to mildly denature the protein and expose the carbohydrate residues. The bottom figure shows the anti-GlcNAc reactivity without the heating step.
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- 15 Figure 2 shows the anti-GlcNAc reactivity of immobilised Sandoglobulin (top) or HIG (bottom). Solutions containing various concentrations of Sandoglobulin of HIG were added to micro titre plates directly/or to plates coated with protein A. Prior to the addition of the anti-GlcNAc monoclonal antibody the protein A coated plates were either heated to 85°C for 10 minutes or incubate at room temperature for a similar period.
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- 25 Figure 3 shows the results of applying Sandoglobulin or HIG (4852) to an affinity column containing immobilised Concanavalin A. Three fractions were obtained: unbound, weakly bound - eluted with 100 mM α -methyl mannoside - and strongly bound - eluted with 100 mM HCl. Various concentrations of each of the three fractions were incubated in protein A coated microtitre wells. The
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anti-GlcNAc reactivity of the immobilised Sandoglobulin of HIG fractions were measured. The bottom figure show the percentage of unbound and bound material for three different HIG preparations and underivatised Sandoglobulin.

Figure 4 shows anti-GlcNAc reactivity of immobilised CLB and derivatised CLB (HIG-62759) and Sandoglobulin and derivatised Sandoglobulin (HIG 4855). Solutions containing various concentrations of the different preparations were added to micro titre plates coated with protein A. The plates were then heated at 85°C for 10 minutes.

Figure 5 shows the anti-GlcNAc reactivity of immobilised Sandoglobulin derivatised with iminothiolane using different buffers. Solutions containing various concentrations of derivatised IgG were added to microtitre plates coated with protein A and then heated at 85°C for 10 minutes.

Figure 6(a) shows how the derivatised antibodies of the invention bind to anti-type II collagen IgG antibodies that are found immobilised in the joint of RA patients. Figure 6(b) shows the difference in the structure of carbohydrate chains in agalactosyl and normal antibodies

Figure 7 shows a schematic drawing of the species involved in the experiments shown in figure 3.

Figure 8 shows schematic drawing explaining how the

results from the stacking assays of figure 2 arise.

Figure 9 shows a graph of extent of denaturation plotted against temperature for HIG and Sandoglobulin, demonstrating how derivatisation leads to a greater degree of denaturation at a given temperature.

Detailed Description

Production of Antibodies

The precursor antibodies may be obtained commercially or prepared using techniques which are standard in the art. Human precursor antibodies can be obtained as commercially available polyclonal mixtures of antibodies such as Sandoglobulin or CLB, or obtained from large universal phage display libraries. Sandoglobulin is a polyclonal IgG/glucose preparation that is produced and commercially available from Sandoz. CLB is a polyclonal IgG preparation which can be obtained from the Central Laboratory of the Blood Transfusion Service of the Netherlands Red Cross, Amsterdam, Netherlands.

Derivatised antibodies made from human precursor antibodies have the advantage that they enable the use of repeat treatments due to the absence of the human anti-mouse antibody (HAMA) response (Schroff et al, Cancer Research, 45:879-885, 1985; DeJager et al, Proc. Am. Assoc. Cancer Res. 29:377, 1988). HAMA responses have a range of effects, from neutralisation of the administered antibody leading to a reduced therapeutic dose, through to allergic responses, serum sickness and renal impairment.

In some instances, antibodies of other species may be suitable precursor antibodies. Methods of producing such antibodies are very well known in the art and include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, Nature, 357:80-82, 1992). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

In some embodiments, the precursor antibodies are monoclonal. Monoclonal antibodies can be made using the hybridoma method first described by Kohler et al, Nature 256:495, 1975, or made using recombinant means. Thus, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Monoclonal antibodies can be subjected to the techniques of recombinant DNA technology to produce other antibodies

or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP 0184187 A, GB-A-2188638 or EP 0239400 A. A hybridoma producing a monoclonal antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

Pharmaceuticals

The antibodies of the invention can be formulated in compositions for therapeutic or diagnostic use. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes, and can be readily selected by those skilled in the art.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has

suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

In therapeutic embodiments, the derivatised antibodies are typically administered in an individual in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980.

Alternatively, targeting properties of the antibodies may be used to deliver the active agent more specifically to locations in a patient's body at which immobilised IgG, e.g. agalactosyl IgG, accumulates.

As mentioned above, the antibodies of the invention can be used to deliver one or more functional moieties to locations associated with immobilised IgG, the functional moiety being provided by the antibody itself or by the conjugation of the antibody to another molecule. An example of the former is the use of an antibody specific for an inflammatory mediator, such as tumour necrosis factor (TNF α). An example of the latter is conjugating the antibody to a precursor form of a drug, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT which involves targeting the activating agent to the cells by conjugation to a cell-specific antibody, see for example EP 0415731 A.

Materials and Methods

1. Production of Monoclonal Antibody 3C4

An IgM monoclonal (3C4) with specificity towards terminal N-acetylglucosamine (GlcNAc) was produced by the UCL Monoclonal Antibody Unit by *in vitro* culture. Initial screening of clones used terminal N-acetylglucosamine rich ovalbumin and then agalactosyl IgG. The 3C4 monoclonal was purified from culture supernatant using GlcNAc-sepharose[®] and eluted with 0.5M GlcNAc. It was then dialysed and biotinylated. This antibody was used as a probe for GlcNAc residues in the experiments described below.

2. 3C4 Stacking Assays

(i) Nunc[®] maxi-sorp plates were coated with 50 μ l of

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Protein A at 2.5mg/ml in PBS overnight at 4°C. Plates were washed with PBS/0.05%Tween[®] 20 (PT) and blocked with 100µl of 1% BSA in PT (PBT) for 1h at 37°C. The plates were then washed with PT, and the human IgG to be tested was added in 100mM glycine/150mM NaCl pH 8.2 buffer as serial five fold dilutions in triplicate starting at 20µg/well in 50µl (400µg/ml) for six dilutions such that the lowest amount was around 6ng/well. The plates were then incubated for 2h at 37°C, and then washed five times with PT. 100µl of PBS was added to each well and the plates were then floated on a water bath at 85°C for 15 minutes to denature the IgG and expose the Fc N-linked oligosaccharides. The PBS was shaken out and 125ng of biotinylated 3C4 monoclonal was then added per well in PBT and incubated overnight at 4°C. Following five further washes visualisation of the bound 3C4 was achieved by addition of 0.1mg 2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulphonic acid] in 50µl 0.1M citrate phosphate, pH 4.1 containing 0.005% hydrogen peroxide, and incubation in the dark for 10 minutes at room temperature. The reaction was stopped by the addition of 50µl of 0.2% sodium fluoride and absorbance measured at 650nm-490nm.

(ii) As a variation on the above assay, human IgG was also coated directly to Nunc[®] Maxi-sorp plates in 50µl PBS at the same serial dilutions 20µg/well to 6ng/well as stated above. Efficient coating was achieved after 2h at 37°C. From this point the assay was identical to the one above from the heating step, i.e. 100µl of PBS was added to each well and the plates were then floated on a water

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bath at 85°C for 15 minutes. This assay gave higher levels of bound human IgG and differs from the previous assay with respect to the orientation of the initial IgG monolayer.

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3. Thiolation of Human IgG

Human IgG (30mg) was dissolved in 0.5ml of 15mM ammonium bicarbonate, with all solutions being prepared and kept oxygen free by using degassed solvent and being maintained under nitrogen. 0.63mg of 2-iminothiolane-HCl in 1 ml of 15mM ammonium bicarbonate was added and mixed gently on a roller device for 20 minutes. The derivatised IgG was then separated from the excess 2-iminothiolane using a disposable Sephadex® G-25 (PD10) column that had been blocked with 1% human serum albumin in 0.9% NaCl, followed by equilibration in 15mM ammonium bicarbonate. The elution of the derivatised human IgG was achieved with 15mM ammonium bicarbonate and monitored by 280nm absorbance. The tin/tartrate solution was made as follows. Di-sodium tartrate (4mg/mg IgG) was dissolved in 10ml of sterile oxygen free water. 4µg of tin (II) chloride per mg IgG was added to the solution (stock solution 750µg Sn²⁺/mol in 1% HCl).

The IgG column eluate was added to the 10 ml solution of tartrate and tin and brought to a final concentration of 1mg IgG/ml using the derivatisation buffer.

4. Preparation of Concanavalin A Positive Human IgG
A 2ml bed volume column of Con A Sepharose® (Sigma) was equilibrated with 50mM Tris pH 7.2 with 200mM sodium

chloride and 2mM MnCl_2 , CaCl_2 . 10mg of human immunoglobulin that had previously been dialysed and freeze dried was dissolved in 1ml of the above buffer and the applied to and then allowed to sit on the column for 10 minutes at room temperature. The column was then washed with several column volumes of the above buffer and the Con A unbound IgG fraction eluted and monitored by 280nm absorbance. The Con A positive fraction was then recovered by elution with 5 column volumes of 100 mM HCl and then the pH was returned back to neutrality as soon as possible with a minimal volume of a 1M Tris solution. The absorbance of the bound fraction was measured at 280nm, it was usually found that approximately 25% of the IgG was Con A positive. Both fractions were dialysed against water and freeze dried prior to further analysis.

Results and Discussion

Figure 1, top, shows that the anti-GlcNAc reactivity of derivatised IgG (HIG) as described in method (3) is greater than the starting material (Sandoglobulin) at any fixed concentration. Since both the Sandoglobulin and the HIG have the same chemical content of terminal N-acetylglucosamine residues on the carbohydrate chains attached to the IgG molecules, respectively, the increase in the reactivity must result from an increase in accessibility of the carbohydrate residues to the 3C4 anti-GlcNAc monoclonal probe.

The carbohydrate residues on the Fc fragment IgG are normally inaccessible since they are buried inside the

protein (see Figure 7). By heating to 85°C, some of the carbohydrates chains become accessible and can then be detected by 3C4. The increase in accessible carbohydrate chains for HIG, when compared to Sandoglobulin, suggests that at 85°C the HIG is more easily thermally denatured, exposing a greater number of chains. This is shown in Figure 9, which shows a graph showing how the accessibility of GlcNAc residues of HIG and the Sandoglobulin starting material varies with temperature. This demonstrates that the derivatisation reaction reduces the temperature at which the carbohydrate chains become available towards physiological temperature (see Figure 1, bottom). This shift is particularly advantageous as it provides a method of derivatising the antibodies while avoiding loss of the antibody binding activity caused by thermal denaturation, e.g. making it possible to make derivatised antibodies that retain a useful activity associated with the antibody binding reaction, such as binding specificity for an inflammatory mediator. The use of thermal denaturation to "flip out" the sugars is not a useful method since (1) on returning to physiological temperatures the sugars will flip back in and (2) the thermal denaturation may disrupt the antigen binding site on the antibody. Accordingly, the two step method for producing derivatised antibodies described in this embodiment provides (1) a chemical denaturant to "flip out" the sugar chains at physiological temperatures followed by (2) a chemical derivatisation to prevent them from flipping back.

When adding increasing amounts of IgG to a microtitre

well saturation would be expected. Figure 2 shows that whereas Sandoglobulin saturates, there is a continual increase in anti-GlcNAc reactivity of the HIG as the concentration of HIG applied to the microtitre plates is increased. This indicates that a new top surface monolayer is being formed for the case of HIG which contains increasing proportions of anti-GlcNAc reactive IgG. This "stacking" of anti-GlcNAc reactive IgG onto the initial Protein A immobilised monolayer and subsequent monolayers is a property unique to the HIG and not found for Sandoglobulin. This phenomenon is described in Figure 8, which show how the proportion of derivatised antibody (shown by the filled circles), as compared to the immobilised IgG (shown by the open circles), increases in successive layers of the stack.

Figure 2 demonstrates that the stacking of anti-GlcNAc reactive IgG also occurs when the initial monolayer is not oriented by the coating of the plate (i.e. Protein A). Figure 2 also demonstrates that even in the absence of heating there is a significant amount of anti-GlcNAc reactive IgG in the HIG preparation and that essentially none exists in the Sandoglobulin.

IgG is also known to contain carbohydrate residues in the Fab region of the molecule. The Fab carbohydrates are accessible to lectin binding and therefore IgG molecules containing Fab associated carbohydrates can be separated by affinity chromatography. Figure 3 (bottom) shows that the percentage of Con A bound (IgG containing Fab carbohydrates) and Con A unbound (IgG with no Fab

carbohydrates) is the same for Sandoglobulin and HIG. Figure 3, top, however shows that all of the "stacking" IgG is found in the Con A bound fraction of HIG. These species are shown schematically in Figure 7. Figure 3 also suggests that in cases in which not all of the derivatised antibodies are capable of stacking, a separation step should be employed to separate the stacking antibodies.

These results suggest that the "stacking" IgG has three specific characteristics:

(a) Firstly, "stacking" HIG is reactive with anti-GlcNAc monoclonal antibodies which are probing the carbohydrates on the Fc fragment.

(b) Secondly, it is only those HIG molecules which also have a Fab associated carbohydrate which stack.

(c) Thirdly, the increased proportion of anti-GlcNAc reactive material which is found when the HIG concentration is increased indicates that the "stacking" IgG prefers to stack itself (i.e. self-association).

In patients with rheumatoid arthritis, there is an increase in the serum content of agalactosyl IgG glycoform. This glycoform is disease associated and is characterised by having an increased proportion of its Fc-associated carbohydrate chains terminating with N-acetylglucosamine rather than galactose or sialic acid, see Figure 6(b). This form of IgG therefore has an

increased anti-GlcNAc reactivity with the 3C4 monoclonal similar to the *in vitro* produced HIG. Immobilised agalactosyl IgG will therefore act as a preferential surface for the "stacking" of HIG *in vivo*. In this way, HIG is able to selectively bind to tissue surfaces (e.g. synovial joints) of patients with autoimmune disorders in the presence of large concentrations of endogenous IgG.

Figures 4 and 5 demonstrate that the iminothiolane derivatisation reaction itself is not sufficient to produce HIG which is able to stack. This result is unexpected and novel. The most probable mechanism is that certain buffers (e.g. ammonium bicarbonate) are able to briefly cause the carbohydrate residues to "flip out", exposing cryptic amino acid residues which can then be derivatised with the iminothiolane. Once derivatised, the presence of the iminothiolane groups sterically prevent the carbohydrate chains from flipping back inside the protein, see Figure 7(b). An alternative mechanism is that when the carbohydrate chains are "flipped out", the iminothiolane derivatisation of amino acid residues remote from the carbohydrate binding site results in stabilisation of a protein conformation in which the carbohydrate chains spend more time "flipped out" at 37°C (see Figure 1(b)). That is, the dynamic equilibrium is shifted such that the sugar chains become more mobile at body temperature and consequently the protein surface becomes exposed for a greater time for interaction with carbohydrate chains from adjacent molecules.

Figure 6(a) shows how the derivatised antibodies of the

invention bind to anti-type II collagen IgG antibodies that are found immobilised in the joint of RA patients. The derivatised antibodies have flipped out carbohydrate residues that are available to bind to the anti-type II collagen IgG antibodies, and have binding sites opened up for binding by the agalactosyl carbohydrate chains of the immobilised IgG. This means that the derivatised antibodies can bind to sites where the immobilised antibodies are located and stack to provide amplification of a label for diagnosis or another kind of effector moiety for a therapeutic purpose.

Figure 6(b) shows the difference in the structure of carbohydrate chains in agalactosyl and normal antibodies, the agalactosyl IgG missing galactosyl and sialic acid residues from the terminus of the carbohydrate chain.

Figure 7 shows a schematic drawing of the species involved in the experiments investigating the stacking properties of the HIG derivatised antibodies described above. Thus, the derivatisation reaction flips out the Fc carbohydrates (compare Figure 7 Con A bound and Figure 7 (HIG)) that were bound to the internal protein surface. This surface (binding site) is now vacant and can interact with another carbohydrate chain on an adjacent IgG molecule. The data in Figure 3 indicates that this carbohydrate chain is the one attached to the Fab (see Figure 7 Con A bound) of an adjacent IgG molecule. Serum agalactosyl IgG (Figure 6(b)) behaves like the derivatised antibodies since it is missing carbohydrate residues exposing the same protein surface which occurs

in derivatised IgG when the carbohydrate chains flip out.

The references mentioned herein are all expressly incorporated by reference.